Clonal Selection, Attenuation and Differentiation in an In Vitro Model of Hyperplasia

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Observations of the growth kinetics and morphologies of clones and subclones of diploid human skin fibroblast cultures lead to the working hypothesis that these cells, presumably like their counterparts in healing wounds, constitute a differentiating system. There is attenuation of the growth of serial clones, with continual selection for more vigorous stem cells. The latter segregate daughter cells of varying growth potential, including a class of cells which may be regarded as terminally differentiating; we propose that such cells may be histiocytes or macrophages. These studies a) demonstrate extensive epigenetic heterogeneity in fibroblast cultures, b) suggest that hyperplastic foci may be monoclonal or oligoclonal, c) rule out a simple biologic clock mechanism as an explanation of clonal senescence, d) suggest a new approach to the analysis of various inborn errors of metabolism, such as Werner's Syndrome (Am J Pathol 74:137–154, 1974).

When fragments of human skin are explanted in culture medium, cells migrate and proliferate from both epidermis and dermis. It seems reasonable to assume that these represent a sampling of cells which would have participated in the healing of the wound had the fragments been reimplanted *in vivo*. To the extent that there may be intrinsic cellular mechanism regulating this proliferation, such control systems might be expected to be expressed *in vitro*. We now report results of experiments consistent with such an expression. The findings have implications for all investigators who use diploid human fibroblasts in their research, since such cultures are shown to be extremely heterogeneous with respect to growth potential and, by implication, various biochemical and physiologic parameters. They also have important implications for gerontologists who use these hyperplastoid cell lines as *in vitro* models for the study of cell senescence.

Materials and Methods

General cell culture methods [1,2] and cloning technics [3,4] have been previously described. The established cultures were cloned at a time when epidermal cells

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were no longer observable; the predominant cell type in actively proliferating mass cultures was fibroblast-like. A modified Dulbecco-Vogt medium, prepared in this laboratory[5] was used throughout, and was supplemented by 16% heat-inactived fetal calf serum. Cultures for *Mycoplasma* (laboratory of G. E. Kenny[6]) were negative. Trypsinized[7] cultures were counted with a hemocytometer. For smaller populations, cells were counted directly with an inverted Nikon phase contrast microscope (Model MS) using a Sage air flow heater to maintain temperature at 37 C (Sage Co, Cambridge, Mass). All lines were established from a standard skin biopsy site (mesial aspect of mid-upper arm). Line 70-48 was from a normal 40-year-old female. Line 69-24 was from a normal male embryo obtained by therapeutic abortion for an indication other than maternal rubella; its gestational age was 63 days. Line 73-15, from a patient (H. McG)[8] with Werner's syndrome was established a few months before her death at age 56. Line 72-80 was obtained from a 36-year-old male at autopsy 9 hours after his death from an intracerebral hemorrhage. The cells used for Figure 1 were (Line 73-16) derived from the skin of a 37-year-old male with trisomy 21. The tantalum phagocytosis experiments were carried out with a line (72-22) derived from autopsy specimens from a 67-year-old female who died after open heart surgery from rheumatic valvular disease. The tantalum (mean particle size = 2 μ) was prepared and the monolayers treated according to the method of Kolodny,[9] except that 5 mg/ml was employed (for 48 hours) and 5 washes were used to remove excess unphagocytized tantalum. (The monolayers used for Figure 3A and B, however, were unwashed.)

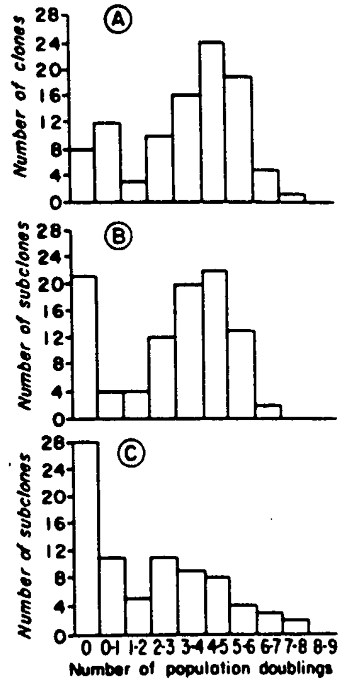
Autoradiography (Figure 2) was carried out on subconfluent monolayers exposed to 0.35 μCi/ml tritiated thymidine (specific activity, 6.7 Ci/mmole) for 24 hours. The slides were coated with NTB II emulsion and developed for 5 days. Fixation was with Carnoy's and staining with hematoxylin and eosin.

For cryobiologic preservation, cells were frozen at a rate of approximately 1 to 2 C minute in complete medium containing 8.3% dimethylsulfoxide and stored in liquid nitrogen; for reconstitution of cultures, they were rapidly thawed at 37 C.

Results

Size Distributions of Clones and Subclones

The histograms of Text-figure 1 illustrate the heterogeneity of the growth of clones and subclones derived from the embryo strain of fibroblasts. Text-figure 1A was generated from a series of 98 primary clones grown from single cells isolated at random from the trypsinized mass culture at a time when it had undergone an estimated 8.5 cumulative population doublings; the mass culture had been reinstituted after cryobiologic preservation in liquid nitrogen. The secondary clones (Text-figure 1B) were derived from the fastest growing primary clone (P12) which had achieved seven to eight population cell doublings in a 5-day period (Text-figure 1A); secondary cloning was carried out when P12 had reached a population of 20,000 cells (fourteen population doublings). In a similar fashion, the best growing secondary and tertiary clones were passaged (after fourteen to fifteen cell doublings each) to generate the distribution of Text-figure 1C, a series of quaternary clones. Thus, at the termination of the experiment, the best of the



TEXT-FIG 1—Distribution of population doublings achieved by clones from strain 69-24. *A*—Primary clones, 5 days after isolation, unfed. *B*—Secondary clones of P12, 5 days after isolation, unfed. *C*—Quaternary clones of P12S81T72 17 days after isolation with feeding on day 9.

slowly growing quaternary clones had a total history of approximately 59 cumulative population cell doublings measured from the time of first passage of the original mass culture. The cumulative population cell doublings of the parental mass cultures was 37.6.

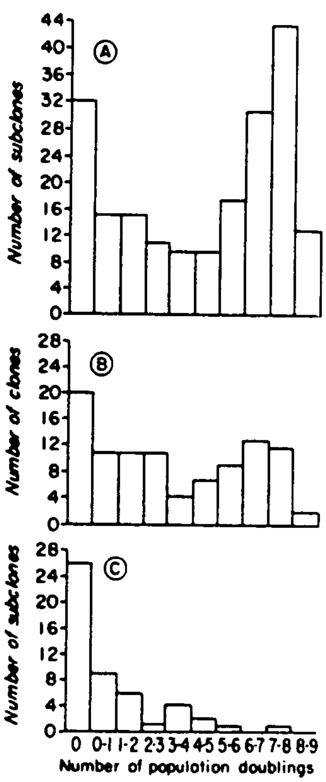
It is clear from the histograms that the growth distributions of clones and subclones were bimodal. One group of cells either did not divide or was capable of only one or two divisions during the experimental periods. Although various interpretations are discussed below, we tentatively classified these cells as "terminally differentiating." Such cells were observed for periods of up to 4 to 5 weeks and did not resume growth. The second group of clones might be regarded as constituting the "proliferative pool" of the cultures, since they continued to grow for varying periods. The statistical distribution of this group appeared to be approximately lognormal,[10] with increasing skewness developing in serial clones.

Several interesting phenomena occurred in serial clones. First of all, a clone selected because of rapid growth clearly segregated daughter cells with a great variety of clonal growth potentials, including terminally differentiating types. With recloning, there was a shift in the distribution of the proliferative pool towards lower growth rates and an increase in the proportion of terminally differentiating cells. The two factors combined to produce a gradual attenuation of growth. This

attentuation was quite striking by the time of quaternary cloning, when it took more than three times as long (17 days vs 5 days) to generate a clone in the range of 7 to 8 population doublings. These subcloning experiments suggest that in cultures of diploid human fibroblasts, there is a continual selection for cells capable of giving rise to the largest number of progeny.

The experiments with the adult strains (Text-figure 2) confirmed the bimodal distributions of clonal growth rates. Text-figures 2A and B are examples of two contrasting subclones from the same parental line, one in which the major peak is found in the proliferative pool, the other (Text-figure 2B) in which most of the plated cells were of the terminally differentiating type. Similar results have been obtained with strain 72-80. With this latter strain, essentially indistinguishable bimodal distributions were generated when versene was elminated from the trypsinization solution.[7]

As expected from our previous experience with mass cultures from patients with Werner's syndrome,[1] primary clonal growth of these cells was very poor (Text-figure 2C). However, once again, a bimodal distribution was noted, and it seemed probable that the perpetuation of this strain was via a very small sample of surviving clones. In a

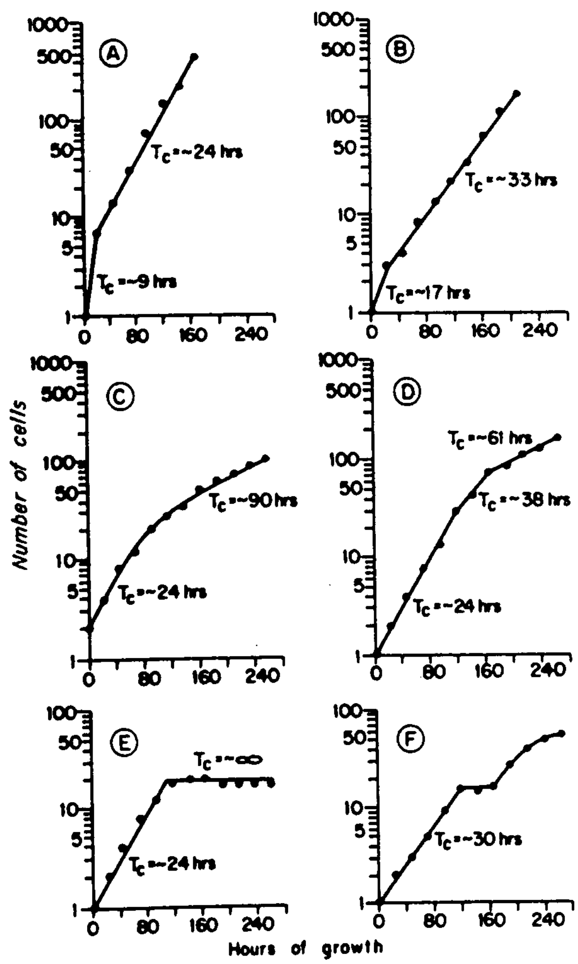


TEXT-FIG. 2—Distributions of population doublings achieved by secondary clones from strain 70-48 9 days after isolation with complete changes of media every 2 days. *A*—Secondary clones of P26. *B*—Secondary clones of P6. *C*—Distribution of population doublings achieved by primary clones from strain 73-15 17 days after isolation with complete changes of media on days 5 and 10.

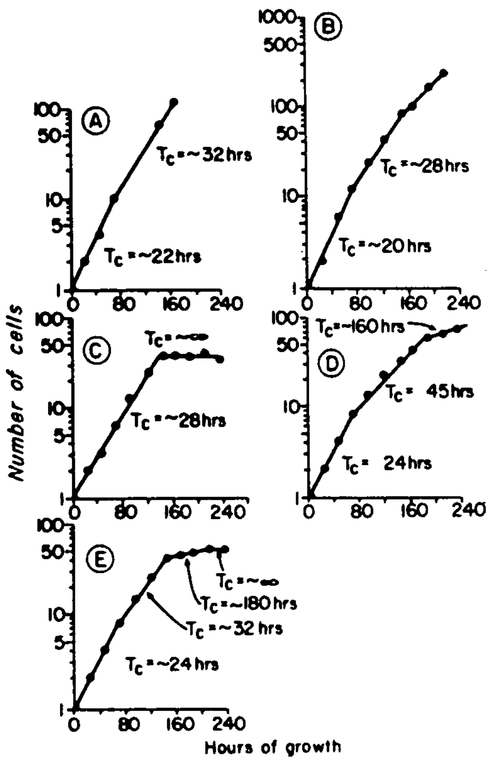
recent experiment in which a normal adult strain (70-48) was *subcloned* for a comparable period of time (17 days), the mode of the proliferative pool (which constituted a larger proportion of the total) was five to six cell doublings (33 to 64 cells), but one subclone at the tail end of the distribution achieved fourteen to fifteen cell doublings (30,000 cells), emphasizing the extreme variability in the number of progeny the cells of a given clone can produce.

Clonal Growth Curves

The heterogeneity in the ultimate sizes of clones was reflected in a heterogeneity of growth rates. Many primary and secondary clones appeared to grow exponentially or nearly exponentially during the first week or so without replenishments of media (Text-figures 3A, B and 4A). If the medium was changed at 2-day intervals and the clone passaged by trypsinization prior to confluency, an occasional primary



TEXT-FIG. 3—Growth curves of clones from strain 70-48. All clones were fed every 2 days. *A*—Primary clones, P6. *B*—Secondary clone P6S14. *C*—Secondary clones P6S66. *D*—Secondary clone P6S9. *E*—Secondary clone P6S6. *F*—Secondary clones P6S32. T_c = mean cell generation time.



TEXT-FIG 4—Growth curves of clones from strain 70-48. All clones were unfed. *A*—Primary clone P1. *B*—Secondary clones P1S28. *C*—Secondary clone P1S10. *D*—Secondary clone P1S48. *E*—Secondary clone P1S30.

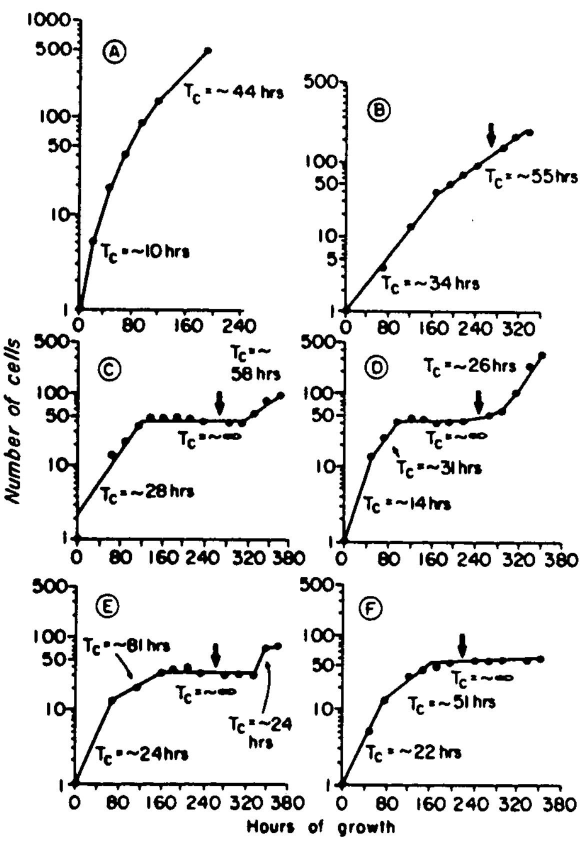
clone grew exponentially or nearly exponentially for at least 3 to 4 weeks. The majority of clones, however, failed to maintain exponential growth during observational periods of about 10 days, with or without media changes. There was great variation with respect to the nature of such growth rate retardations among cohorts of subclones (Text-figures 3 and 4). While in some cases comparatively smooth curves could be drawn on semilog plots indicative of a gradual change in growth rate (Text-figure 3C), in most cases there appeared to be sharp discontinuities in such plots. The first discontinuity was sometimes hard to evaluate, since the initiating cells could have been isolated at various points in the mitotic cell cycle. However, in some cases a decreasing series of sharp discontinuities in growth rates were observed (Text-figures 3D, F, 4B, D and E). Relatively sudden cessations of growth were commonly observed (Text-figures 3E and 4C). Only rarely would growth resume after what appeared to be a cessation or marked slowing of growth in cultures fed every other day (Text-figure 3F). The responses of *unfed* subclones to a single feeding (day 11) varied. Actively growing subclones showed no apparent change in growth rate (Text-figure 5B). Some subclones which had ceased to grow resumed growth, but after variable postfeeding lag periods (Text-

figures 5C, D and E). Finally, there was a class of subclones which did not resume growth after feeding (Text-figure 5F).

It should be emphasized that while, rarely, the initial growth rate of a subclone appeared to surpass that of the parental clone (Text-figure 4A and B), there was always subsequent attentuation of growth such that the ultimate yield of cells of a subclone was never greater than that of the parental clone during comparable periods of observations.

Morphologic Observations

The cells of clones which we have operationally referred to as terminally differentiating were larger and more epithelioid than those of the proliferative pool. However, comparable large epithelioid cells with irregular pseudopods could frequently be identified within actively proliferating clones. Occasionally, one could directly visualize a highly asymmetric division, with a very large nondividing daughter cell and a smaller sister cell capable of further replication (Figure 1A). Clusters of terminally differentiating cells are shown in Figure 1B; their cyto-



TEXT-FIG 5—Growth curves of clones from strain 69-24. *Arrow* indicates the point of media changes. *A*—Primary clone P12. *B*—Secondary clone P12S100. *C*—Secondary clone P12S8. *D*—Secondary clone P12S48. *E*—Secondary clone P12S1. *F*—Secondary clone P12S56.

plasms sometimes appear to contain an abundance of filamentous structures. Most cell divisions were characterized by lesser degrees of asymmetry as regards the comparative dimensions of the daughter cells. It was also the rule that the subsequent cell divisions of daughter cells were to some extent asynchronous.

In preliminary studies with living and stained monolayers of mass cultures, many cells have been observed to apparently phagocytize red blood cells, bacteria, conidia, tantalum, cotton fibers and cellular debris. The amount of material taken up varies substantially among cells of given monolayers. It is our impression that there is a greater degree of phagocytosis by the large, phenotypically "old" or "terminally differentiated" cells which predominate in late passage cultures (Figure 2A–C). In early passage cultures, nonreplicating cells appear to be more highly phagocytic, although it is clear that some cells which incorporate thymidine take up significant amounts of tantalum. Quantitative studies of phagocytosis have not yet been carried out with either clones or mass cultures.

Cell-to-cell contacts were observed during the first few days of growth, long before confluence (Figure 1C). These were sometimes via long, thin, apparently tubular cellular extensions, which we refer to as "intercellular tunnels." Such structures have been observed to rupture spontaneously and then to subsequently regenerate (Figure 3). We believe they may be characteristic of the terminally differentiating type of cell, since, as in the case of Figure 3, such cells either do not replicate or replicate slowly.

When vigorous clones were allowed to develop without trypsinization for 3 to 4 weeks ("megaclones"), then fixed and stained, they demonstrated a characteristic histologic structure. Discrete regions composed of dense multilayerings of cells were periodically reproduced—either as radiating straight "ribs" (Figure 1D) or as "whirls" (Figure 1E). No such apparent organization was observed with megaclones of L cells, a line derived from mouse subcutaneous tissue, or with megaclones of Chang liver cells, an epithelioid cell line.

Discussion

Our laboratory has previously published evidence consistent with the participation of more than one type of differentiated cell in the constitution of early mass hyperplastoid cultures derived from human skin.[11,12] More recently, we have also commented on the presence of "smooth muscle"-type clones in such cultures.[13] Therefore, it seemed important to us that, in any comparative clonal analysis of the growth

properties of the major cell types, the growth of *subclones* was investigated. We are aware of unpublished studies of the replicative life-spans of WI-38 primary clones, however, which do indicate bimodality.[14] In our previous studies[1] on the replicative life-spans of clones of neonatal foreskin fibroblasts, the methods were such that we were clearly selecting for study a small proportion (approximately 10%) of isolated cells, namely those with the best growth, so that the striking heterogeneity reported in this paper was not appreciated. The real situation was first perceived by Merz and Ross.[15] In the present experiments, bimodal distributions of growth have been observed in both primary clones and their derivative subclones (Text-figures 1 and 2). The frequencies of any minor cell types in uncloned mass cultures, such as "smooth muscle" cells,[13] are presumably insufficient to grossly alter the statistical distributions when sample sizes are of the order of 50 to 200.

There are various interpretations of this bimodality, the most trivial of which is that it represents some sort of artifact, for example a bimodal distribution of coverslips, toxic and nontoxic. Other potential artifacts include mechanical injuries during cell isolation or chemical injuries, attributable to trypsin and/or versene. This interpretation appears unlikely, unless one proposes that the cells of older cultures and cultures from patients with Werner's Syndrome are more susceptible to such injuries, and, moreover, that the response to such agents is bimodal. The most suspicious potentially injuries agent, versene (0.55 mM), has been ruled out as a significant variable. The data is hard to explain by any conventional formulation of protein error theory,[16,17] as one would not expect such striking and reproducible patterns of heterogeneity. One would have to postulate a mechanism whereby there was preferential segregation of abnormal proteins and abnormal protein-synthetic machinery to one of two daughter cells. A type of unbalanced growth associated with unequal distributions of cytoplasmic organelles and/or of cell membrane remains a possibility, especially if one considers the probable importance of cell surface receptor sites for growth-promoting factors in the media.[18] We prefer a differentiation hypothesis, however, for a variety of reasons, none of which could be regarded as compelling at the present stage of our research. First of all, the notion of a fibroblast to macrophage transformation has a certain teleologic appeal. Fibroplasia and and phagocytosis are two essential features of healing wounds; it seems reasonable that evolution might have selected for a mesenchymal cell which could segregate daughter cells capable of sustained replication and the synthesis of

structural macromolecules such as collagen, as well as daughter cells with highly developed phagocytic functions. Secondly, there is the striking experimental observation, at least in *Drosophila,* that *prior* to the initiation of differentiation, normal somatic cells (imaginal discs) may be cultured indefinitely.[19] Thirdly, there is ample precedent for such a model in biology. The best studied system involves hematopoiesis.[20] Recent work with this model does in fact suggest different degrees of "stemness" among populations of hematopoietic stem cells.[20,21] Furthermore, a type of clonal senescence has been reported for this system.[22] Clonal senescence has also been reported in an *in vivo* lymphoid cell model;[23] these investigators currently favor a differentiation model to explain their results.[24] In the hematopoietic model, one of course has the advantage of remarkable morphologic differentiations as well as characterized marker proteins, such as hemoglobin. Although less striking, we believe that there is morphologic differentiation occurring in our cultures and that the presumptively differentiating cells have a range of morphologies consistent with those of histiocytes or macrophages. Even the observations of cytoplasmic filamentous structures (Figure 1B) might be reconciled as aggregates of contractile microfilaments.[25] Light microscopic observations suggestive of a transformation of fibroblasts into macrophages are as old as the art of tissue culture.[26,27] Observations by more modern investigations are also consistent with this interpretation. Several electron microscopists have observed increasing proportions of enlarged lysosome-rich cells with increasing age of culture.[28,29,30] These cells are rich in acid phosphatase and other lysosomal enzymes.[30,31] They have been interpreted as fibroblasts which have undergone "senescence" *in vitro.* Such cells may in fact undergo degenerative changes, but only as a secondary postreplicative event. Abnormal proteins[32,33] might well accumulate in such degenerating "macrophages" on a posttranslational basis. The failure to find such "senescent fibroblasts" in skin biopsies of aged adults[28] was taken as evidence that the *in vitro* phenomenon was an inappropriate model for the study for the ageing of connective tissue cells. Had these workers studied biopsies of healing *wounds* in subjects of various ages, it is possible that they may have found closer parallels. On the basis of our tissue culture experience, one would predict a greater proportion of lysosome-laden macrophages in the healing wounds of older subjects. Our preliminary morphologic observations really add very little to establish the identity of these putatively differentiated cells as macrophages. The crucial experiments will require immunocytochemical technics for the identification of surface receptors

thought to be characteristic of macrophages.[34,35] Of course, it is possible that in our system we are dealing with a phylogenetically primitive nonimmunologic phagocytic defense mechanism and that more sophisticated lines of macrophages were independently evolved. The key element in support of a differentiation hypothesis, regardless of what one calls the differentiated progeny, would be the demonstration of differential gene expression. The error theory predicts abnormal proteins. The differentiation theory predicts a different spectrum of normal proteins.

That the decline of cultured somatic cells is related in some way to biologic aging is strongly supported by our published age regression data.[1] It is also supported by the findings with biopsies from patients with Werner's syndrome, who suffer from an acceleration of a variety of pathologic processes commonly associated with aging.[8] The present findings with clones from a patient with Werner's syndrome further document the restrictive replicative life-spans of such cultures. In addition, they suggest to us a new point of view as regards the general nature of the basic defect. It is possible that in such patients there is an excessive rate, premature onset or defective type of differentiation within the proliferative pools of certain cell types during growth and cell turnover. Indeed, the failure to realize the normal adolescent growth spurt despite an apparently normal endocrine environment is the earliest and most striking feature of the disease.[8] An aberrancy in the control of mesenchymal cell growth and differentiation is also suggested by the high incidence of mesenchymal neoplasms in these patients.[8]

Our differentiation model is of further heuristic value in that it predicts the expression, in skin fibroblast cultures, of inborn errors of metabolism involving a cell type or cell types other than the "fibroblast." For example, if it can be established that the putative differentiating cells are macrophages, then it would be of great interest to investigate phagocytic functions in cultures from a variety of patients with unusual susceptibilities to infectious disease, including diabetics.

We are entirely ignorant of the ultimate molecular mechanisms responsible for the observed attentuation of clonal growth. Our subcloning experiments rule out any simple form of biologic clock theory whereby cells are thought to have a mechanism for counting the number of cell divisions, replication ceasing at some predetermined number programmed by the genome.[36] The use of the word "differentiation" certainly does not solve our problem; since there is little understanding of the regulation of normal differentiation *in vivo*, it is difficult to pro-

pose any molecular model to explain why cultures might "differentiate themselves to death" *in vitro*. Potential regulatory interaction among cells appears to us as a fruitful area for further study. We are impressed with the evidence of growth control at the population level (Text-figures 3–5). Intercellular communication and metabolic cooperation are well developed in hyperplastoid cell lines [37,38] and appear to have a structural basis [38] (Figures 1C–E and 3)—further evidence, in our view, that we are dealing with a differentiating system. Morphologic observations of developing clones [13] and the apparent histology of megaclones (Figure 1D and E), are also suggestive of some sort of differentiation; similar periodic regions of dense cellular multilayering have been observed in mass cultures of fibroblasts and have been related to local production of collagen.[39] Periodicities in growth rates do occur in mass cultures [13] and could be mediated by perturbations in the equilibrium created by positive and/or negative feedback signals between proliferating and terminally differentiating cells.[40] Our findings of an approximately 60% increase in the cumulative population doublings of serial clones compared to parental mass cultures is consistent with some type of negative feedback control. However, such a result might simply be a consequence of the dilution, in mass cultures, of a small replicating pool by large numbers of nonreplicating or slowly replicating cells; the cumulative population doublings is based upon an averaging of both classes of cells.

We have recently summarized the many contrasting properties of hyperplastoid and neoplastoid cell lines and have suggested that the former might serve as models for the study of certain aspects of hyperplasia or wound healing. The working assumption is that basic aspects of growth regulation observed *in vitro* also apply *in vivo*. Thus, since there appears to be continual selection in our cultures for clones capable of contributing large numbers of progeny, we tentatively conclude that healing wounds might be comprised predominately of only one or a few clones. One should therefore be cautious about equating evidence for monoclonicity with evidence for mutation or transformation.[41] On the other hand, there is no assurance that the degree of clonal selection we have observed in our cloning experiments also occurs in mass cultures, let alone in healing wounds *in vivo*. In any case, it is probable that, *in vivo*, the growth kinetics are subject to important modulations by a variety of environmental factors.

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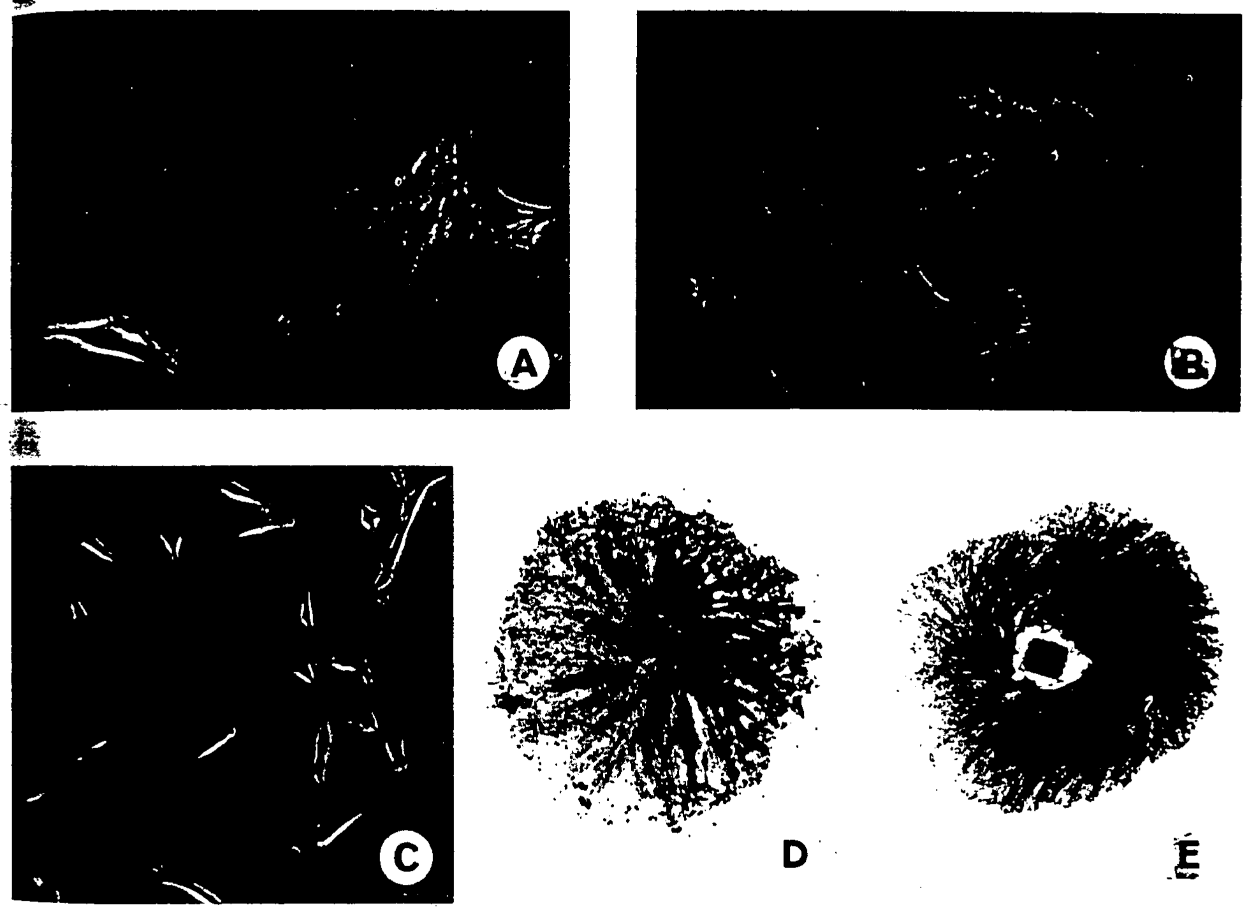

Fig 1A—Asymmetric division of a single cell 24 hours after isolation. Secondary clone of strain 70-48 (P17S28) (Phase contrast microscopy, × 396). **B**—Cluster of terminally differentiating cells in a primary clone (P41) strain 70-48 (Phase contrast, × 318). **C**—Intercellular contacts between cells in a subconfluent, actively replicating primary clone. Strain 72-80, day 5 (Phase contrast, × 286). **D**—Megaclone, strain 69-24. Fixed and stained 30 days after growth from a single cell (dilute plating on 2 × 3 inch glass slides). The irregular stippling around the periphery is an artifact of precipated stain (1% Crystal violet in 20% ethanol, × 2.24). **E**—Megaclone, strain 73-16. Fixed and stained 4 weeks after cell growth from a coverslip (3 cu mm) inoculum of 10,000 cells. The cell-free zone around the 3-cu mm cover slip is an artifact related to parital retraction of the "mono-layer" (× 1.65).

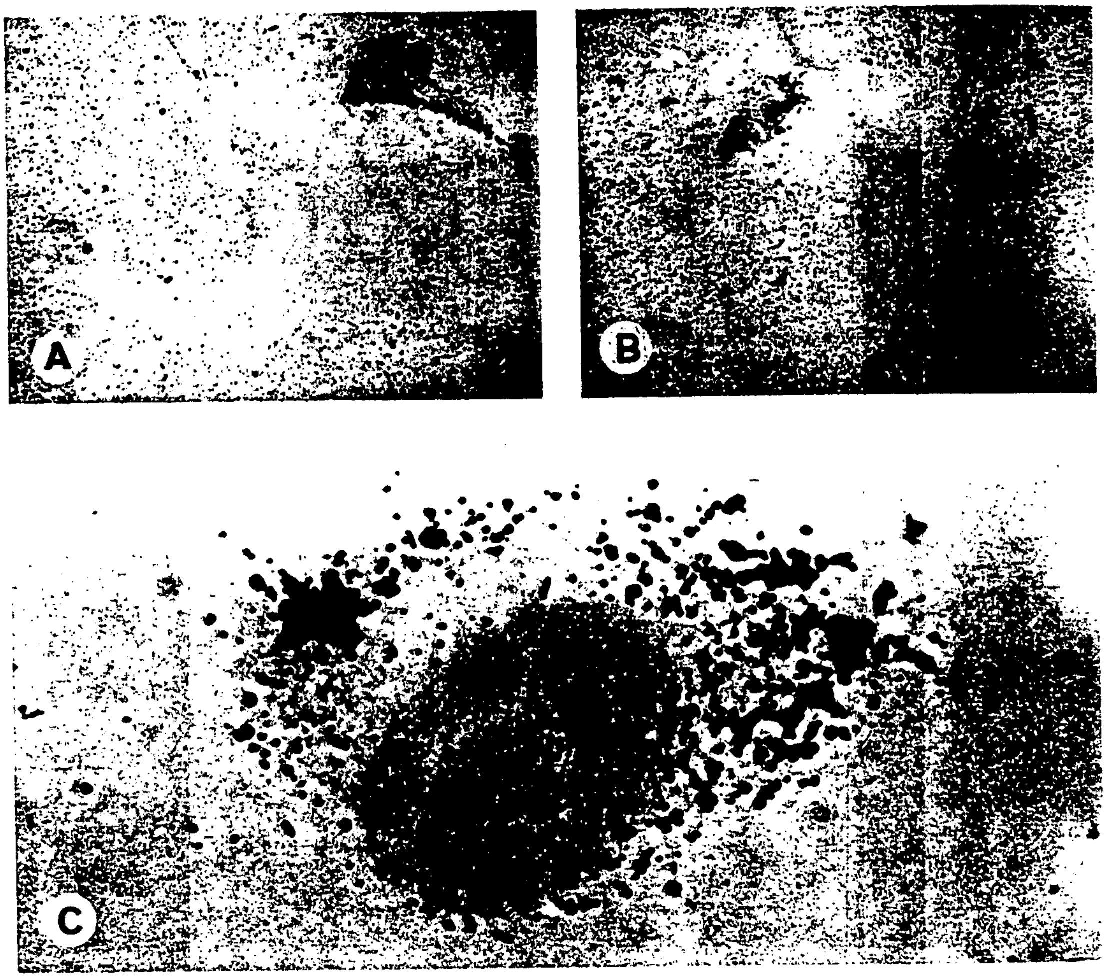

Fig 2—Phagocytosis of autoclaved tantalum by mass cultures of human skin fibroblasts (line 72-22). **A and B**—"Tracks" from which tantalum has been cleared by motile phagocytic cells of a late passage culture in which only 1.4% of nuclei incorporated tritiated thymidine during a 24-hour pulse (× 80). **C**—Phenotypically "old" or terminally differentiated cell from comparable late passage mass culture (× 1000).

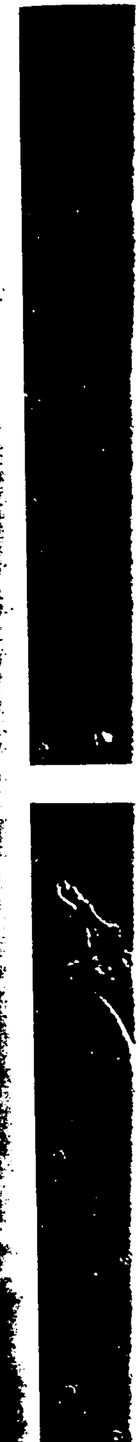

Fig 3
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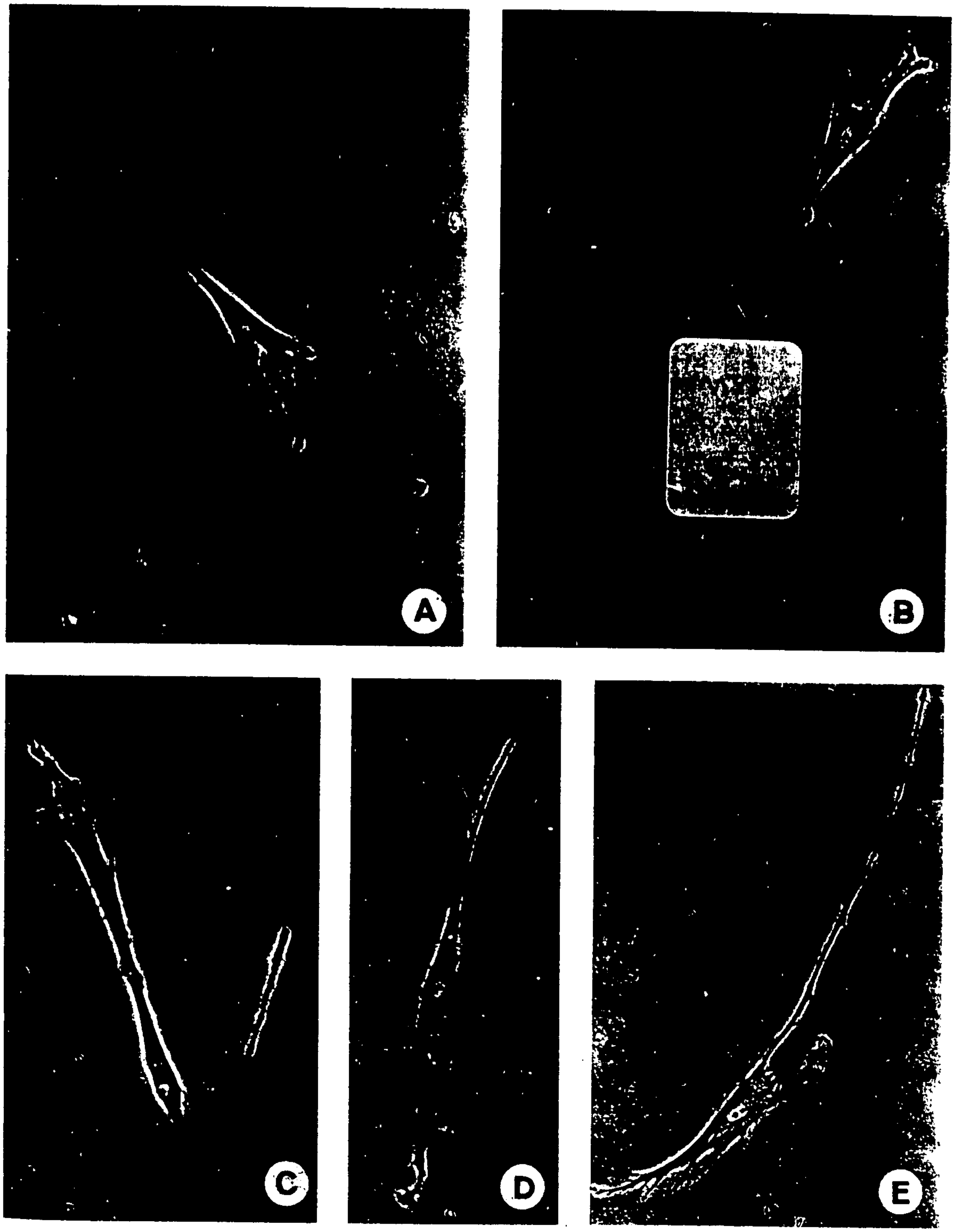

Fig 3—Time sequence of a single living terminally differentiated cell showing formation, breakage and subsequent reformation of an "intercellular tunnel." A similar or identical process is thought to be characteristic of macrophages and has been referred to as "clasmatosis." [42] Such projections have been seen to bridge two or more widely separated cells. Strain 70-48, Phase contrast microscopy. A—Twenty-four hours (× 348)). B—Forty-eight hours (× 331). C—Seventy-two hours (× 412). D—Ninety-six hours (× 329). E—One hundred twenty hours after isolation from mass culture by trypsinization (× 281).

[*End of Article*]